DETERMINATION OF α-AMYLASE ACTIVITY IN FLUE-CURED TOBACCO USING A CHROMOGENIC SUBSTRATE

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The activity of α-amylase in flue-cured tobacco was determined using a sensitive chromogenic substrate. In analysis of an NC 95 tobacco sample, more enzyme activity was found following flue-curing than was present in the green leaf. Studies of the effect of pH on flue-cured tobacco enzyme activity showed a double pH optima at pH 5.8 and pH 6.25. The α-amylase present in tobacco might be utilized in tobacco processing.

INTRODUCTION

Information on amylase activity present in tobacco during processing is of interest because tobacco quality is enhanced by starch conversion to sugars. In the presence of sufficient enzyme, the alteration of conditions, such as temperature and moisture, might be utilized to enhance enzyme activity and improve the product or decrease processing time.

Gaines and Meudt (5) reported starch contents ranging from one to five percent in cured leaf of Georgia grown flue-cured tobacco. There have been conflicting reports on amylase activity in cured tobacco possibly because of the methods of enzyme extraction and analysis used. Barrett (1) found no amylase activity in unaged flue-cured bright and unaged air-cured burley tobacco. Spencer and Weston (11) studied α-amylase activity in *Nicotiana tabacum* (var. Hicks' Broadleaf) leaf discs. Activity was reported to increase rapidly during the first few hours of flue-curing, then to decrease, with the activity in cured leaves slightly lower than that in green tobacco. Levels of α-amylase in green and flue-cured NC 95 tobacco determined by a new, sensitive chromogenic substrate for the enzyme assay are contained in this report. The dyed amylose, which has just recently been developed and used for determination of sweet potato and human salivary α-amylases (3), does not react with β-amylase. Modification of internal portions of an amylose chain hampers hydrolysis by β-amylase (12). Alpha amylase is thought to operate by a front-side and beta amylase by a back-side displacement reaction (13). Steric congestion caused by the dye bound to the amylose could block accessibility of the binding sites and prevent reaction of β-amylase.

MATERIALS AND METHODS

Nicotiana tabacum from flue-cured tobacco variety NC 95 was grown at Oxford, North Carolina in 1972. A portion was freeze dried while green and the remainder was flue-cured and freeze dried. Stems were removed from both samples and the tobacco was ground in a Wiley Mill. South Carolina grown flue-cured tobacco (1971) was obtained from Universal Leaf Tobacco Company,[2] Richmond, Virginia. This redried sample was freeze dried prior to enzyme analysis. The enzyme extract was prepared at 1 to 5°C. A 5 g freeze dried tobacco sample was ground to powder in a chilled mortar, extracted 30 sec with a Vir Tis 45 homogenizer in 75 ml phosphate buffer (0.04 M, pH 6.2, containing 0.001 M $CaCl_2$) in the presence of 3.8 g polyvinyl pyrrolidone (PVP) (7, 8), and filtered through two layers of cheese cloth. The extraction was repeated using 50 ml phosphate buffer, and the combined extract centrifuged for 10 min at 2500 rpm, 5°C. Protein was precipitated from the supernatant by adding $(NH_4)_2SO_4$ to 80% saturation (9). After 30 min at 2°C, the mixture was centrifuged, the precipitate suspended in phosphate buffer, and dialyzed at 5°C against the buffer until sulfate free. The nondialyzable material was centrifuged, the supernatant retained, and the precipitate washed with buffer and recentrifuged. The combined supernatant was diluted to 50 ml, and frozen. The sample was diluted to a measurable range prior to enzyme assay. Extracts of the NC 95 green and flue-cured tobacco were prepared in duplicate.

Synthesis of the substrate previously reported by Dougherty (3), involved reaction of amylose with the dye Cibachron Blue F3GA. The complex formed was treated with $CaCl_2$ to produce a substrate which showed much greater reactivity with tobacco α-amylase than a commercially available dyed amylose (3).

Synthesis of Substrate. A solution of 12.5 g Cibachron Blue F3GA in 1.25 l. H_2O was heated to 60°C. Amylose (125 g) was gradually mixed with 500 ml H_2O, then an additional 750 ml H_2O was added, and the mixture heated to 60°C. The dye was added to the amylose, followed by 370 g Na_2SO_4 gradually added over a 25 min period. A solution of 12.5 g Na_3PO_4 in 180 ml H_2O (60°C) was added, and heating at 60°C continued for an additional 75 min with slow mechanical stirring.

The mixture was cooled at room temperature, centrifuged for 15 min at 15°C, 3,000 rpm. The dyed amylose was washed with water until the supernatant was pH 6 to 7. The residue was washed with MeOH-H_2O (800-800 ml) and with one l. MeOH, centrifuged, then incubated in water at 50°C for 3¾ hr, centrifuged, and the supernatant decanted. The dyed amylose was incubated at 44°C for 1½ hr in 0.04 M phosphate buffer (pH 6.2), containing 0.001 M $CaCl_2$, centrifuged and washed with water until the washings were practically colorless. The substrate was stirred with a glass rod until the particles formed a suspension and freeze dried.

α-Amylase Assay. Tobacco enzyme extract (0.2 ml) was incubated with dyed amylose substrate (70 mg) in 2.3 ml of 0.04 M phosphate buffer, pH 6.25, containing 0.001 M $CaCl_2$ at 37°C for 15 min. The reaction was terminated by addition of 4.5 ml of 0.1 acetate buffer, pH 3.5. The absorbance of the supernatant at 625 nm was read against a blank (without enzyme). Reaction of dilute enzyme extracts with 20 ± 0.5 mg substrate in 1.0 ml incubation volume and 4.2 ml final volume was linear with activity levels below 0.35 absorbance. Phosphate buffer adjusted to various pH values was used for determination of pH optima, except at points below pH 5.8, where phthalate—NaOH buffer was employed. Enzyme activity units (per g freeze dried tobacco) were calculated by reference to a calibration curve constructed as described by Ewen (4) from aliquots of a Cibachron

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[2] *Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.*

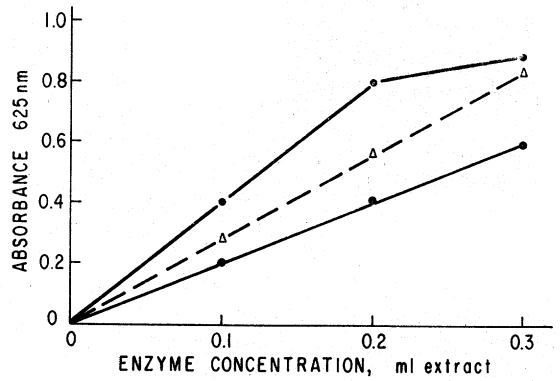

Figure 1. Linearity of amylase activity with increasing enzyme concentration of green (△) and flue-cured (0) tobacco extracts.

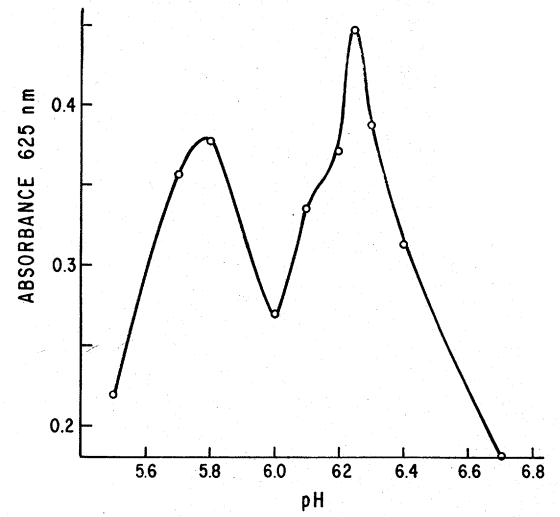

Figure 2. Effect of pH on flue-cured tobacco enzyme hydrolysis of dyed amylose.

Blue F3GA solution (0.200 g dye/l.) diluted with acetate buffer. One unit of enzyme activity is defined as the amount of enzyme which results in liberation of 0.01 micromole of dye per minute under the conditions defined for assay. The molecular weight of the dye is 773.5; therefore, in a 15 min incubation period, a sample with an activity of 1 unit per aliquot would liberate $\frac{773.5 \times 15}{100} = 116$ μg of dye.

RESULTS AND DISCUSSION

Enzyme activity was linear with concentration up to but not beyond an absorbance of 0.836 **(Figure 1)**, and duplicates agreed ±0.008 absorbance units at the highest level, and ±0.004 units generally. Reaction rates were linear for at least 20 min. The NC 95 tobacco serial samples were found to have an α-amylase activity of 241.5 units/g (240.5 and 242.5 units/g, duplicate extracts) in the green tobacco and 483.8 units/g (512.5 and 455.0 units/g) after flue-curing. The 1971 South Carolina flue-cured and redried sample contained 170 units/g.

pH Optima. **Figure 2** illustrates the effect of pH on enzyme activity in South Carolina flue-cured and redried tobacco. The double pH optima observed at pH 5.8 and pH 6.25 may indicate isoenzymes with different pH optima. Electrophoretic studies, which could confirm the presence of isoenzymes, were not conducted. Pressey (10) reported a biphasic curve for potato invertase in the presence of an inhibitor; therefore, influence of an inhibitor active below pH 6.25 should be considered. Peaks at pH 5.8 and pH 6.25 in approximately equal proportion were obtained similarly with the NC 95 flue-cured sample which was assayed at less than half the enzyme concentration used in analysis of the South Carolina tobacco. The pH curve for green tobacco was not obtained. Spencer and Weston (11) observed only one tobacco amylase peak at pH 6.2; however, their enzyme extract preparation and assay procedure (iodine) were different, and their paper does not state whether the sample analyzed was green or flue-cured tobacco. The extraction procedure employed in the present report included use of PVP to remove phenolics, which can inhibit amylase (8), and protein precipitation with $(NH_4)_2SO_4$ to partially purify the enzyme. If the presence of isoenzymes with different pH optima is confirmed, studies directed toward determination of changes in α-amylase activity during curing would require assay at both of the pH optima.

Since greater α-amylase activity was present in flue-cured than in green NC 95 tobacco, the enzyme activity might be utilized in guiding modifications of curing procedures to improve quality or decrease processing time, e.g., by regulating temperature and moisture for optimal α-amylase activity in order to obtain desired reducing sugar content (2, 6). The presence of starch in tobacco is thought to provide protection to the enzyme against heat inactivation during flue-curing (11). It is possible that redrying leads to partial inactivation of α-amylase; however, no conclusions can be drawn from the single sample of South Carolina flue-cured and redried tobacco.

Amylase activity found in green and flue-cured tobacco could be influenced by the tobacco samples, curing conditions, enzyme extraction and assay procedures. Enzyme inactivation during preparation of the enzyme extract may account for the absence of amylase activity reported by Barrett (1). The presence of inhibitors manifested during flue-curing may be responsible for the lower enzyme activity following flue-curing found by Spencer and Weston (11), who used a relatively large concentration of tobacco extract in their assay. Very dilute enzyme sample and a sensitive substrate were used in the present study. Although the iodine used in previous studies could react with other substances present in tobacco extracts, the assay described here is specific for α-amylase; β-amylase does not react with the substrate. In analysis of sweet potato juice, excellent correlation was obtained between the AOAC method for α-amylase and this procedure (3).

The dyed amylose substrate provides a convenient, sensitive method for determining α-amylose in tobacco. Information obtained using the enzyme assay should be useful in guiding modifications of tobacco processing, e.g., studies could be conducted on the relationship between tobacco α-amylase activity and the time required to attain particular reducing sugar levels under controlled conditions of temperature and moisture. Such information may be useful in production of homogenized leaf, where conditions for optimal α-amylase activity might be maintained for a period of time relative to the enzyme level, and thereby obtain desired reducing sugar content.

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